Three years of alendronate treatment results in similar levels of vertebral microdamage as after one year of treatment

Matthew R. Allen[1] and David B. Burr[1,2,3]

[1]Departments of Anatomy and Cell Biology and [2]Orthopaedic Surgery, Indiana University School of Medicine

[3]Biomedical Engineering, Indiana University-Purdue University at Indianapolis

Funding sources: NIH Grants AR047838 and AR007581.

Running title: Alendroante and microdamage

Email: matallen@iupui.edu; dburr@iupui.edu

Send Correspondence to:

Matthew R. Allen, PhD

Dept. of Anatomy and Cell Biology, MS 5035

Indiana University School of Medicine

635 Barnhill Dr.

Indianapolis, IN 46202

Tel: 317-274-1283

FAX: 317-278-2040

Email: matallen@iupui.edu

Number of words (characters) in abstract: 407 (2,791)
Number of words (characters) in manuscript: 2,913 (16,924)
Number of figures: 3
Number of tables: 4

Three years of daily alendronate treatment increases microdamage in vertebral bone but does not significantly increase it beyond levels of microdamage found after 1 year of treatment. This suggests microdamage accumulation peaks during the early period of bisphosphonate treatment, and does not continue to accumulate with longer periods of treatment.

Introduction: Clinically-relevant doses of alendronate increase vertebral microdamage by 4- to 5-fold in skeletally mature beagles after 1 year of treatment. The goal of this study was to determine if microdamage would continue to accumulate with three years of alendronate treatment in an intact beagle dog model.

Methods: One-year-old female beagles were treated with daily oral doses of vehicle (VEH, 1 ml/kg/day) or alendronate (ALN, 0.2 mg/kg/day or 1.0 mg/kg/day) for three years. These ALN doses were chosen to approximate, on a mg/kg basis, those used to treat osteoporosis (ALN0.2) and Paget's disease (ALN1.0). Microdamage accumulation, static and dynamic histomorphometry, densitometry, and mechanical properties of lumbar vertebrae were assessed. Comparisons were made among the three groups treated for three years, and also within each treatment group, to animals treated under the same conditions for one year (Allen et al. Bone, 2006).

Results: Overall microdamage accumulation (crack surface density) was not significantly higher in animals treated for three years with either dose of ALN, while crack density increased significantly (100%; $p < 0.05$) with the higher dose of ALN when compared to VEH. Both ALN doses significantly suppressed the rate of bone turnover (-60% versus VEH). There was no difference among groups for any of the structural biomechanical properties - ultimate load, stiffness, or energy absorption. However, when adjusted for areal bone mineral density ALN-treated animals had significantly lower energy absorption (-20%) compared to VEH. Toughness, the energy absorption capacity of the bone tissue, was significantly lower than VEH for both ALN0.2 (-27%) and ALN1.0 (-33%). Compared to animals treated for one year, there was no significant difference in microdamage accumulation for either ALN dose. VEH-treated animals had significantly lower bone turnover (-58%) and significantly higher levels of microdamage (+ 300%) compared to values in 1 year animals. Toughness was significantly lower in animals treated for 3 years with ALN1.0 (-18%) compared to animals treated for 1 year while there was no difference in toughness between the two treatment durations for either VEH or ALN0.2.

Conclusions: Although three years of alendronate-treatment resulted in higher microcrack density in vertebral trabecular bone compared to control dogs, the amount of microdamage was not significantly higher than animals treated for 1 year with similar doses. This suggests that bisphosphonate-associated increases in microdamage occur early in treatment. Because toughness continued to decline significantly over three years of treatment at the higher ALN dose, decreases in toughness are probably not dependent on damage accumulation.

INTRODUCTION

Microdamage accumulates with age [1,2] and may play an important role in age-associated bone fragility [3,4]. Microdamage formation occurs in response to mechanical loads [5-7], preferentially at sites of increased tissue mineralization [8-10], and is removed by remodeling [6,7]. The level of skeletal microdamage is determined by the balance between microdamage formation and its removal. Therefore conditions that either increase microdamage formation, or decrease its removal, can have a significant impact on the accumulation of microdamage and bone fragility.

Bisphosphonates are efficacious for reducing fractures due to their suppression of bone remodeling [11-13]. However, as reductions in remodeling are permissive for the accumulation of microdamage, bisphosphonate treatment also increases skeletal microdamage. Numerous animal studies have noted significant increases in microdamage following bisphosphonate treatment [14-18]. This accumulation of microdamage occurs with alendronate and risedronate doses comparable to those used for the treatment of post-menopausal osteoporosis, although the accumulation is greater when higher doses are given (e.g. those approximating doses used for treatment of Paget's disease) [18].

Whether microdamage accumulation continues or plateaus with extended bisphosphonate treatment is not known, yet has significant implications as some patients now enter their second decade of treatment. Studies to date have assessed microdamage at a single time point, most often one year. Recently, Komatsubara et al. [16,17] reported that 3 years of daily incadronate, at 2.5 or 5x the clinical dose, significantly increased the accumulation of microdamage in both the vertebrae and rib of dogs. As no data are available concerning microdamage levels with shorter term incadronate treatment (< 3 years), this study was not able to address whether microdamage accumulation continues or plateaus with prolonged bisphosphonate treatment.

Animal studies documenting increased microdamage with bisphosphonate-treatment have consistently shown increases in vertebral bone strength and stiffness, leading to questions regarding the implications of increased microdamage with bisphosphonates. However, in all but one of these studies [16] bisphosphonate treatment reduced bone toughness, the energy

absorption capacity of the bone tissue [14,15,17,18]. Furthermore, when normalized for increases in BMD, energy absorption capacity at the whole bone (structural) level was significantly compromised following 1 year of alendronate treatment [19]. If microdamage continues to accumulate with prolonged bisphosphonate treatment it is possible that this could lead to further reductions in work to failure and toughness.

The goal of this study was to test the hypothesis that microdamage continues to accumulate throughout the duration of bisphosphonate treatment and that this continued accumulation is accompanied by a progressive decline in energy absorption and toughness. We have recently documented that clinically-relevant doses of alendronate reduce vertebral bone turnover by more than 70%, increase microdamage by 4- to 5-fold and non-significantly reduce vertebral toughness by 14-17% in skeletally mature beagles after 1 year of treatment [18]. The current study reports results from animals treated for three years with the same doses of alendronate used in the one year study. This allows both an across-treatment analysis at the three-year time point (vehicle versus alendronate) as well as a within-treatment analysis across time points (1 versus 3 years).

MATERIALS AND METHODS

Animals

All procedures were approved prior to the study by the Indiana University School of Medicine Animal Care and Use Committee. Thirty-six female beagles (1-2 years old upon arrival) were purchased from LBL (Reelsville, IN). Upon arrival, lateral X-rays of all dogs were obtained to confirm skeletal maturity (closed proximal tibia and lumbar vertebra growth plates). Animals were housed two per cage in environmentally controlled rooms at Indiana University School of Medicine's AALAC accredited facility and provided standard dog chow and water. Two dogs (both in the ALN 0.2 group) developed hernias, both in year 2, that required surgery. One of these animals developed a second hernia which progressed to the point of needing to be terminated early (month 34 of treatment); this animal was still included in all analyses. All other animals completed the 36 month treatment without serious complication.

Experimental Design

Following two weeks of acclimatization, animals were assigned to treatment groups (n=12/group) by matching body weights. All dogs were treated daily for 3 years with oral doses of vehicle (saline, 1 ml/kg/day) or alendronate sodium (0.20 or 1.00 mg/kg/day; Merck and Co., Inc). Alendronate doses were chosen to approximate, on a mg/kg basis, the doses used for treatment of post-menopausal osteoporosis and Paget's disease, respectively. Alendronate was dissolved in saline and administered to the dogs orally with a syringe. Vehicle-treated animals received 1 ml/kg/day of saline. Dosing was performed each morning after an overnight fast and at least 2 hours prior to feeding.

Prior to necropsy, animals were injected with calcein (0.20 mL/kg, IV) using a 2-12-2-5 labeling schedule. Animals were euthanized by intravenous administration of sodium pentobarbital (0.22mg/kg Beuthanasia-D Special). After death, lumbar vertebrae were dissected and saved for analyses. The second and third lumbar vertebrae were fixed in 10% neutral buffered formalin while the fourth lumbar vertebra was wrapped in saline-soaked gauze and frozen (-20ºC). All tissue preparation, processing, and analyses were similar to those used for dogs treated for one year [18].

Histology (Static, dynamic, and microdamage)

Static and dynamic histomorphometric measures of trabecular bone were obtained on second lumbar vertebrae (L2). Bones were embedded undecalcified in methyl methacrylate (MMA; Aldrich). Mid-sagittal (4 µm) sections were cut using a Reichert-Jung 2050 microtome (Magee Scientific, Inc) and stained with McNeal's tetrachrome for static histomorphometry. Mid-sagittal (8 µm) sections were cut and left unstained for dynamic histomorphometry and wall thickness measures.

Third lumbar vertebrae (L3) were processed for microdamage assessment by bulk staining in basic fuchsin as previously described [18,20]. Using 1% basic fuchsin dissolved in increasing concentrations of ethanol, specimens were stained according to the following schedule: 8 hours 80% (with one change to fresh 80% after 4 hours), overnight in 95% (with one change to fresh

95%), 8 hours in 100% (with one change to fresh 100% after 4 hours). Bones were placed under vacuum (20 in Hg) for all stages during the day and left on the bench top overnight. Following staining, bones were washed in 100% ethanol and embedded undecalcified in MMA. Mid-sagittal (80-100 µm) sections were cut using a diamond wire saw (Histosaw; Delaware Diamond Knives).

Histological measurements were made using a semiautomatic analysis system (Bioquant OSTEO 7.20.10, Bioquant Image Analysis Co.) attached to a microscope equipped with an ultraviolet light source (Nikon Optiphot 2 microscope, Nikon). A 5 x 5 mm region of interest, located 1 mm below the cranial plateau, was used for sampling. Static and dynamic variables were measured and calculated in accordance with ASBMR recommended standards [21]. Microdamage was assessed using UV fluorescence as previously described [22]. Measurements included crack length (Cr.Le, µm) and crack number (Cr.N), with calculations of crack density (Cr.Dn, #/mm^2; Cr.N / bone area) and crack surface density (Cr.S.Dn, µm/mm^2; Cr.N * Cr.Le / bone area).

Densitometry

Areal bone mineral density (aBMD, g/cm^2) of the fourth lumbar vertebra (L4), without the posterior elements or cranial/caudal endplates, was quantified using a PIXImus II densitometer (Lunar Corp.). Volumetric bone density and geometry of the L4 vertebra was quantified using a Norland Stratec XCT Research SA+ pQCT (Stratec Electronics). One slice (0.07 X 0.07 x 0.50 mm voxel size) was taken at three locations (25, 50 and 75% of total vertebra height). Total, trabecular, and cortical volumetric bone mineral density (vBMD, mg/cm^3) and cross-sectional area (CSA, mm^2) were obtained for each slice and then averaged together to obtain a single representative value for each specimen.

Biomechanical Testing

The biomechanical properties of L4 vertebrae were quantified using a servohydraulic testing system (MTS Bionix, MTS Corporation). Compression to failure was carried out on saline soaked specimens using displacement control mode (20 mm/min). Load vs displacement data were

digitally recorded at a sampling rate of 10Hz. Plots were analyzed for determination of ultimate force (F), stiffness (k) and work to ultimate force (w). Apparent material-level properties ultimate stress (σ_{ult}), modulus (E), and toughness (U) were estimated using the following equations: σ_{ult} = (F / CSA) / BV/TV; E = (k * (height / CSA)) / BV/TV; U = (w / (height * CSA)) / BV/TV, where cross sectional area (CSA) is from pQCT, height measured using digital calipers, and BV/TV from L2 histomorphometry.

Statistics

All statistical tests were performed using SAS software (SAS Institute, Inc.). To determine whether variables were different among treatment groups after 3 years, data were evaluated using a one-way analysis of variance (ANOVA) with Fisher's protected least-significant difference (PLSD) post-hoc tests. Strength-density and energy absorption-density relationships from three-year treated animals were compared between VEH and ALN treatments using analyses of covariance with least square means (LSM) used to determine differences in parameters after accounting for aBMD. To determine whether changes occurred within treatment groups across time, t-tests were used to compare data from animals treated for three years with results from an earlier study in our lab which treated animals under the same conditions for one year [18]. For all tests, $p \leq 0.05$ was considered statistically significant. All data are presented as mean ± standard error.

RESULTS

At the conclusion of the study there was no significant difference in body mass among the three groups (VEH: 12.6 ± 0.6 kg; ALN0.2: 12.4 ± 0.5 kg; ALN1.0: 11.6 ± 0.7 kg; p = 0.492).

Crack density, the number of microcracks per mm bone tissue, was significantly higher than VEH for ALN1.0 (+100%, p = 0.01), but not ALN0.2 (+50%; p = 0.12)(Figure 1A). Mean crack length was significantly smaller in both ALN-treated groups compared to VEH (-20% for both) (Figure 1B). Crack surface density, the product of crack density and crack length, was not significantly different among groups (Figure 1C).

Activation frequency (Ac.f) was significantly lower than VEH in both ALN0.2 (-59%) and ALN1.0 (-60%) treated animals. The reduction in Ac.f resulted from significant suppression of both mineral apposition rate (MAR) and mineralizing surface (MS/BS), with no change in wall thickness. MAR was 17% lower than VEH for both doses of ALN while MS/BS was -51% and -62% for ALN0.2 and ALN1.0 groups, respectively (Table 1).

Structural biomechanical properties – ultimate load, stiffness, and energy to ultimate load – were not significantly different among the treatment groups (Table 2). When normalized for aBMD, there was no difference in the strength-density relationship between VEH- and ALN-treated animals (Figure 2A). The slope of the energy absorption-density relationship was similar between treatments yet at a given aBMD the energy absorption capacity was significantly lower in vertebrae from ALN-treated animals (-20%, $p = 0.01$) compared to VEH (Figure 2B). For both the strength-density and energy absorption-density relationships the two doses of ALN were pooled as the results were similar when doses were assessed separately.

Toughness, the energy absorption capacity of the bone tissue, was significantly lower in both ALN0.2 (-26%) and ALN1.0 (-33%) groups compared to VEH (Table 2). There was no difference among groups for the other two material-level properties, ultimate stress and modulus.

Vertebral aBMD was not significantly different among groups while vBMD tended to be higher ($p=0.056$) in both ALN0.2 and ALN1.0 groups (both +7%) versus VEH (Table 3). Trabecular vBMD, cortical vBMD, and cross-sectional area were not different among the three treatment groups. Trabecular bone volume, assessed by histology, was significantly greater in both ALN0.2 (+23%) and ALN1.0 (+31%) treatment groups compared to VEH (Table 3).

After three years of treatment Ac.f. was significantly lower in ALN0.2 (-40%, $p = 0.01$), but not ALN1.0 (-30%, $p = 0.30$), compared to similar treatment groups at 1 year (Figure 3a). The level of microdamage (both Cr.Dn and Cr.S.Dn) was not significantly different at 3 years compared to 1 year for either ALN group (Figure 3b, 3c). Compared to 1-year of treatment, ALN0.2 had higher ultimate load (+21%), stiffness (+55%) and modulus (+65%) at 3 years while ALN1.0 had significantly higher stiffness (+42%) and modulus (+30%) and lower toughness (-18%) (Table 4 and Figure 3). VEH-treated animals had significantly lower Ac.f. (-58%), higher

microdamage accumulation (+301%), and higher structural- and material-level strength and stiffness at 3 years compared to VEH-treated animals after 1 year (Table 4 and Figure 3).

DISCUSSION

Animal studies have consistently documented higher levels of microdamage in bisphosphonate-treated animals [14-18] yet it has remained unclear whether microdamage accumulation continues or plateaus with extended bisphosphonate treatment. Recently, we have documented that clinically-relevant doses of alendronate increase microdamage by 4- to 5-fold in skeletally mature beagles after 1 year of treatment [18]. We now present data to show that the level of microdamage in vertebral trabecular bone does not significantly increase with an additional two years of alendronate treatment (3 years total treatment duration) at doses approximating those used to treat post menopausal osteoporosis or Paget's disease.

As remodeling is necessary to remove microdamage [6,7], bisphosphonate-treatment would be expected to allow accumulation of damage due to turnover suppression. While the degree of turnover suppression is correlated to the degree of microdamage accumulation [15,17,18], even mild suppression of turnover (~40%) with bisphosphonate-treatment is sufficient to allow significant increases in microdamage [18]. The current study shows that the initial suppression of turnover with bisphosphonate treatment has the greatest influence on microdamage accumulation. Following one year of ALN-treatment, vertebral bone turnover is suppressed by ~70%, associated with a 4 to 5-fold increase in microdamage [18]. With an additional 2 years of treatment, and a continued decline in turnover (-30 to -40% compared to values in 1 year animals), microdamage was not significantly increased (1.3- and 1.6-fold higher than VEH). The most plausible explanations for this finding are 1) microdamage can be controlled at a new equilibrium level even with only 30% of normal bone turnover and/or 2) there is a reduced formation of microdamage. The latter could result from the lowering of trabecular strains due to the 20-30% increase in bone volume (Table 3).

Consistent with the relationship between turnover suppression and microdamage accumulation, animals treated for 3 years with vehicle had significantly lower turnover (-58%) and

significantly higher levels of microdamage (+300%) compared to those treated for 1 year. These data highlight that microdamage accumulation is not due to bisphosphonates, per se, but rather the reduction in turnover brought about by bisphosphonate treatment.

Toughness, the energy absorption capacity of the material, is consistently reduced in bisphosphonate-treated animals [14,15,17,18]. This change has often been attributed to microdamage accumulation although a cause and effect has yet to be established. The current results provide two pieces of evidence to suggest microdamage accumulation is not 'causing' reduced toughness in bisphosphonate-treated bone. First, despite higher levels of microdamage in VEH-treated animals after three years (compared to 1 year), there was no change in bone toughness. Second, despite no significant difference in microdamage accumulation between animals treated for one and three years with either dose of ALN, animals treated with ALN1.0 had significantly lower bone toughness at three years compared to one year. While these data do not disprove a cause/effect relationship they strongly suggest bisphosphonate-associated reductions in bone toughness extend beyond simply the accumulation of microdamage.

Structural biomechanical properties – ultimate load, stiffness, and energy absorption – were not significantly different than VEH after 3 years of ALN treatment. These results differ from those at one year, where both doses of ALN significantly increased vertebral stiffness [18] and the higher dose of ALN increased strength [15]. The absence of difference among these groups treated for 3 years is likely the result of significant increases in VEH-treated animals, which had significantly higher ultimate load (+24%) and stiffness (+68%) compared to values in 1 year treated animals. These higher structural-level mechanical properties in 3-year VEH-treated animals compared to 1-year VEH-treated animals likely result from age-associated periosteal expansion. Vertebral cross-sectional area, which plays a significant role in determining structural parameters and results from continued periosteal expansion, was significantly higher (+16%) in the 3-year VEH group compared to the 1 year group. Material-level properties – ultimate stress and modulus – were also higher in VEH-treated animals at 3 years compared to one year. We have recently documented increases in collagen cross-linking and collagen maturity of vertebrae that are attributable to turnover suppression [23]. As the organic matrix is known to affect

material properties, we hypothesize that the reduction in turnover between 1 year and 3 years in vehicle-treated animals (-58%) results in an increase in collagen cross-linking and maturity which, in conjunction with other parameters such as mineralization and microdamage, determine material-level biomechanical properties [24].

An alternative approach to investigate the effects of bisphosphonate treatment on biomechanical properties is to compare the relationships between bone density and biomechanical properties. Proposed by Hernandez and Keaveny [25], these relationships allow the determination of changes in bone strength or energy to fracture that are not accounted for by a change in bone mass (aBMD). ALN-treated animals had 20% lower energy absorption capacity at a given aBMD, indicating that an increase in BMD is necessary with alendronate treatment to maintain energy absorption capacity at a level comparable to non-treated bone. This result is consistent with the 22% lower energy absorption at a given aBMD following one year of treatment with doses of ALN approximating those used to treat osteoporosis [19].

Given the invasive nature of both microdamage and biomechanical property measures, it proves difficult to determine if the changes noted in the current study extend to humans treated with bisphosphonates. Higher levels of microdamage exist in bisphosphonate-treated women [26], although there is no data to support whether there exists a similar treatment-duration accumulation pattern as noted in the current study. Bisphosphonates have clear anti-fracture efficacy suggestive of improved biomechanical properties [11-13]. However, given the multi-factorial nature of fractures it remains possible that reduced toughness or lower energy absorption at a given aBMD could exist even in light of an overall population reduction in fracture risk with bisphosphonates. Indeed both toughness and energy absorption are compensated for by the increased bone density that routinely occurs with bisphosphonate treatment, but the material properties of the tissue are nevertheless compromised.

In conclusion, three years of alendronate-treatment resulted in higher microcrack density in vertebral trabecular bone of intact beagle dogs, yet the amount of microdamage was not significantly higher than in animals treated with equivalent doses for 1 year. This suggests that increased skeletal microdamage associated with turnover suppression occurs early in treatment,

and does not progress with longer treatment duration. Because toughness continued to decline significantly over three years of treatment at the higher ALN dose, decreases in toughness are probably not dependent on damage accumulation.

ACKNOWLEDGEMENTS

The authors thank Keith Condon, Diana Jacob, and Lauren Waugh for histological preparation and Andrew Koivuniemi and Mark Koivuniemi for assistance with densitometry and mechanical testing. This work was supported by NIH Grants AR047838 and AR007581 and utilized an animal facility constructed with support from Research Facilities Improvement Program Grant Number C06 RR10601-01 from the National Center for Research Resources, National Institutes of Health.

342 FIGURE LEGENDS

343 Figure 1. Microdamage parameters in vertebral trabecular bone following three years of daily
344 vehicle (VEH) or alendronate (ALN) treatment (0.20 or 1.00 mg/kg/day). (A) Crack density, the
345 number of microcracks normalized to bone area, was significantly higher ($p = 0.032$) in animals
346 treated with the higher dose of alendronate (ALN1.0). (B) Mean crack length was significantly
347 lower ($p = 0.013$) with both doses of ALN. (C) Crack surface density, the product of crack
348 density and crack length, was not significantly different among groups ($p = 0.149$). There was no
349 significant difference between doses of ALN for any microdamage parameter. * $p < 0.05$ versus
350 VEH.

351

352 Figure 2. Strength-density (A) and energy absorption-density (B) relationships of vertebral bone
353 from beagles treated for 3 years with vehicle (VEH) or alendronate (ALN). Areal bone mineral
354 density (aBMD) was assessed by densitometry while strength and energy absorption were
355 assessed by monotonic compression biomechanical tests. The strength-density relationship was
356 similar for vehicle (○, $y = 20397x - 2065$) and alendronate-treated animals (●, $y = 23385x - 3033$).
357 The slope of the energy absorption-density relationship was similar yet the intercepts differed
358 significantly between vehicle (○, $y = 9912x – 1306$) and alendronate-treated animals (pooled (●),
359 $y = 11489x – 2228$). After adjusting for aBMD, the energy absorption capacity was significantly
360 lower (-20%) in ALN-treated specimens compared to VEH. ALN-treated groups were combined
361 as there was no difference between the two doses for either relationship.

362

363 Figure 3. Differences in activation frequency (A) crack density (B) and toughness (C) between
364 animals treated for 1 and 3 years with vehicle (VEH) or alendronate (ALN0.2 and ALN1.0). (A)
365 Activation frequency was significantly lower in both VEH and ALN0.2 after 3 years of treatment
366 compared to animals at 1 year. (B) Crack density, the number of microcracks normalized to
367 bone area, was not significantly different for either dose of ALN but was significantly higher in
368 VEH-treated animals after 3 years compared to animals treated for 1 year. (C) Toughness, the
369 material-level energy absorption capacity, was significantly lower in animals treated with the

higher dose of alendronate (ALN1.0) after 3 years compared to values at 1 year. * $p < 0.05$ versus 1 year animals within treatment.

REFERENCES

1. Frost HM 1960 Presence of microscopic cracks in vivo in bone. Bull Henry Ford Hosp 8:417-423.
2. Schaffler MB, Choi K, Milgrom C 1995 Aging and matrix microdamage accumulation in human compact bone. Bone 17(6):521-25.
3. Burr DB, Forwood MR, Fyhrie DP, Martin RB, Schaffler MB, Turner CH 1997 Bone microdamage and skeletal fragility in osteoporotic and stress fractures. J Bone Miner Res 12(1):6-15.
4. Donahue SW, Galley SA 2006 Microdamage in bone: implications for fracture, repair, remodeling, and adaptation. Crit Rev Biomed Eng 34(3):215-71.
5. Burr DB, Martin RB, Schaffler MB, Radin EL 1985 Bone remodeling in response to in vivo fatigue microdamage. J Biomech 18(3):189-200.
6. Bentolila V, Boyce TM, Fyhrie DP, Drumb R, Skerry TM, Schaffler MB 1998 Intracortical remodeling in adult rat long bones after fatigue loading. Bone 23(3):275-81.
7. Mori S, Burr DB 1993 Increased intracortical remodeling following fatigue damage. Bone 14(2):103-9.
8. Parfitt AM 1993 Bone age, mineral density, and fatigue damage. Calcif Tissue Int 53 Suppl 1:S82-5; discussion S85-6.
9. Norman TL, Wang Z 1997 Microdamage of human cortical bone: incidence and morphology in long bones. Bone 20(4):375-9.
10. Wasserman N, Yerramshetty J, Akkus O 2005 Microcracks colocalize within highly mineralized regions of cortical bone tissue. Eur J Morphol 42(1-2):43-51.
11. Bone HG, Hosking D, Devogelaer J-P, Tucci JR, Emkey RD, Tonino RP, Rodriguez-Portales JA, Downs RW, Gupta J, Santora AC, Liberman UA, the Alendronate Phase III Osteoporosis Treatment Study Group 2004 Ten Years' Experience with Alendronate for Osteoporosis in Postmenopausal Women. N Engl J Med 350(12):1189-1199.
12. Black DM, Cummings SR, Karpf DB, Cauley JA, Thompson DE, Nevitt MC, Bauer DC, Genant HK, Haskell WL, Marcus R, Ott SM, Torner JC, Quandt SA, Reiss TF, Ensrud KE 1996 Randomized trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. Fracture Intervention Trial Research Group. Lancet 348(9041):1535-41.
13. Cummings SR, Black DM, Thompson DE, Applegate WB, Barrett-Connor E, Musliner TA, Palermo L, Prineas R, Rubin SM, Scott JC, Vogt T, Wallace R, Yates AJ, LaCroix AZ 1998 Effect of alendronate on risk of fracture in women with low bone density but without vertebral fractures: results from the Fracture Intervention Trial. JAMA 280(24):2077-82.
14. Mashiba T, Hirano T, Turner CH, Forwood MR, Johnston CC, Burr DB 2000 Suppressed bone turnover by bisphosphonates increases microdamage accumulation and reduces some biomechanical properties in dog rib. J Bone Miner Res 15(4):613-20.
15. Mashiba T, Turner CH, Hirano T, Forwood MR, Johnston CC, Burr DB 2001 Effects of suppressed bone turnover by bisphosphonates on microdamage accumulation

and biomechanical properties in clinically relevant skeletal sites in beagles. Bone 28(5):524-31.

16. Komatsubara S, Mori S, Mashiba T, Li J, Nonaka K, Kaji Y, Akiyama T, Miyamoto K, Cao Y, Kawanishi J, Norimatsu H 2004 Suppressed bone turnover by long-term bisphosphonate treatment accumulates microdamage but maintains intrinsic material properties in cortical bone of dog rib. J Bone Miner Res 19(6):999-1005.
17. Komatsubara S, Mori S, Mashiba T, Ito M, Li J, Kaji Y, Akiyama T, Miyamoto K, Cao Y, Kawanishi J, Norimatsu H 2003 Long-term treatment of incadronate disodium accumulates microdamage but improves the trabecular bone microarchitecture in dog vertebra. J Bone Miner Res 18(3):512-20.
18. Allen MR, Iwata K, Phipps R, Burr DB 2006 Alterations in canine vertebral bone turnover, microdamage accumulation, and biomechanical properties following 1-year treatment with clinical treatment doses of risedronate or alendronate. Bone 39(4):872-9.
19. Allen MR, Burr DB In Press Changes in vertebral strength-density and energy absorption-density relationships following bisphosphonate treatment in beagle dogs. Osteoporos Int.
20. Burr DB, Stafford T 1990 Validity of the bulk-staining technique to separate artifactual from in vivo bone microdamage. Clin Orthop Relat Res (260):305-8.
21. Parfitt A, Drezner M, Glorieux F, Kanis J, Malluche H, Meunier P, Ott S, Recker R 1987 Bone histomorphometry: Standardization of nomenclature, symbols, and units. Journal of Bone and Mineral Research 2(6):595-610.
22. Huja SS, Hasan MS, Pidaparti R, Turner CH, Garetto LP, Burr DB 1999 Development of a fluorescent light technique for evaluating microdamage in bone subjected to fatigue loading. J Biomech 32(11):1243-9.
23. Gineyts E, Allen MR, Burr DB, Delmas PD 2006 Effects of antiresorptive therapy on the bone tissue concentration of enzymatic mature collagen crosslinks and pentosidine. J Bone Miner Res 21 (Supp 1):M344.
24. Allen MR, Burr DB 2007 Mineralization, Microdamage, and Matrix: How Bisphosphonates Influence Material Properties of Bone. BoneKEy 4(2):49-60; http://www.bonekey-ibms.org/cgi/content/abstract/ibmske;4/2/49.
25. Hernandez C, Keaveny T 2006 A biomechanical perspective on bone quality. Bone 39(6):1173-81.
26. Stepan JJ, Burr DB, Pavo I, Sipos A, Michalska D, Li J, Fahrleitner-Pammer A, Petto H, Westmore M, Michalsky D, Sato M, Dobnig H 2007 Low bone mineral density is associated with bone microdamage accumulation in postmenopausal women. Bone:doi:10.1016/j.bone.2007.04.198.

Table 1. Dynamic histomorphometry of the second lumbar vertebrae

	Vehicle 1 ml/kg/day	Alendronate 0.20 mg/kg/day	Alendronate 1.00 mg/kg/day	p value
MAR, um/day	1.36 ± 0.08	1.13 ± 0.06 *	1.11 ± 0.07 *	0.028
MS/BS, %	7.63 ± 0.99	4.15 ± 0.51 *	3.30 ± 0.92 *	0.002
BFR/BS, $\mu m^3/\mu m^2$/year	36.75 ± 0.10	16.70 ± 0.04 *	14.31 ± 0.09 *	0.0004
Ac.f, #/year	0.793 ± 0.097	0.328 ± 0.037 *	0.319 ± 0.094 *	0.0002

N= 12 animals per treatment group. MAR, mineral apposition rate; MS/BS, mineralizing surface per unit bone surface; BFR/BS, bone formation rate normalized to bone surface; Ac.f, activation frequency. * $p < 0.05$ vs vehicle.

Table 2. Compressive biomechanical properties of the fourth lumbar vertebrae

	Vehicle 1 ml/kg/day	Alendronate 0.20 mg/kg/day	Alendronate 1.00 mg/kg/day	p value
Ultimate Load, N	4656 ± 234	4966 ± 254	4847 ± 304	0.710
Stiffness, N/mm	11889 ± 1153	14241 ± 1146	13622 ± 956	0.299
Energy to Ultimate Load, Nmm	1961 ± 198	1764 ± 140	1581 ± 136	0.260
Ultimate Stress/(BVTV)	1.78 ± 0.11	1.64 ± 0.14	1.51 ± 0.07	0.229
Modulus/(BVTV)	67.3 ± 6.9	74.1 ± 9.1	62.9 ± 3.2	0.518
Toughness/(BVTV)	0.049 ± 0.004	0.036 ± 0.003 *	0.033 ± 0.002 *	0.004

N= 12 animals per treatment group. BV/TV, bone volume normalized to tissue volume. * $p < 0.05$ vs vehicle.

Table 3. Lumber vertebrae bone mineral density, geometry, and bone volume

	Vehicle 1 ml/kg/day	Alendronate 0.20 mg/kg/day	Alendronate 1.00 mg/kg/day	p value
Whole aBMD, g/cm^2	0.330 ± 0.010	0.343 ± 0.011	0.337 ± 0.009	0.644
Total vBMD, mg/cm^3	554 ± 14	591 ± 13	597 ± 12	0.056
Trabecular vBMD, mg/cm^3	329 ± 7	349 ± 7	341 ± 6	0.111
Cortical vBMD, mg/cm^3	1020 ± 10	1019 ± 7	1027 ± 6	0.746
CSA, mm^2	136 ± 4.5	131 ± 5.3	127 ± 5.1	0.461
Trabecular BV/TV, %	19.9 ± 1.3	24.5 ± 1.7 *	25.8 ± 1.7 *	0.029

N= 12 animals per treatment group. aBMD, areal bone mineral density; vBMD, volumetric bone mineral density; CSA, cross sectional area; BV/TV, bone volume normalized to tissue volume.
* $p < 0.05$ vs vehicle.

Table 4. Percent difference between animals treated for 1 and 3 years within treatment

	Vehicle 1 ml/kg/day	Alendronate 0.20 mg/kg/day	Alendronate 1.00 mg/kg/day
aBMD, g/cm^2	-1	-5	-4
CSA, mm^2	***+17***	***+15***	***+15***
Ultimate Load, N	***+24***	***+21***	+14
Ultimate Stress/(BVTV)	***+20***	+18	-1
Stiffness, N/mm	***+68***	***+55***	***+42***
Modulus/(BV/TV)	***+62***	***+65***	***+30***
Energy to Ultimate Load, Nmm	+13	+7	-3

Comparisons between parameters of lumbar vertebrae after 1 and 3 years of treatment (1 year data from Allen et al, Bone 2006). Values represent percent difference between 1 and 3 year animals. ***Bold*** denotes significance ($p < 0.05$). N= 12 animals per treatment group per time point. aBMD, areal bone mineral density; CSA, cross sectional area; BV/TV, bone volume normalized to tissue volume.

Figure 1.

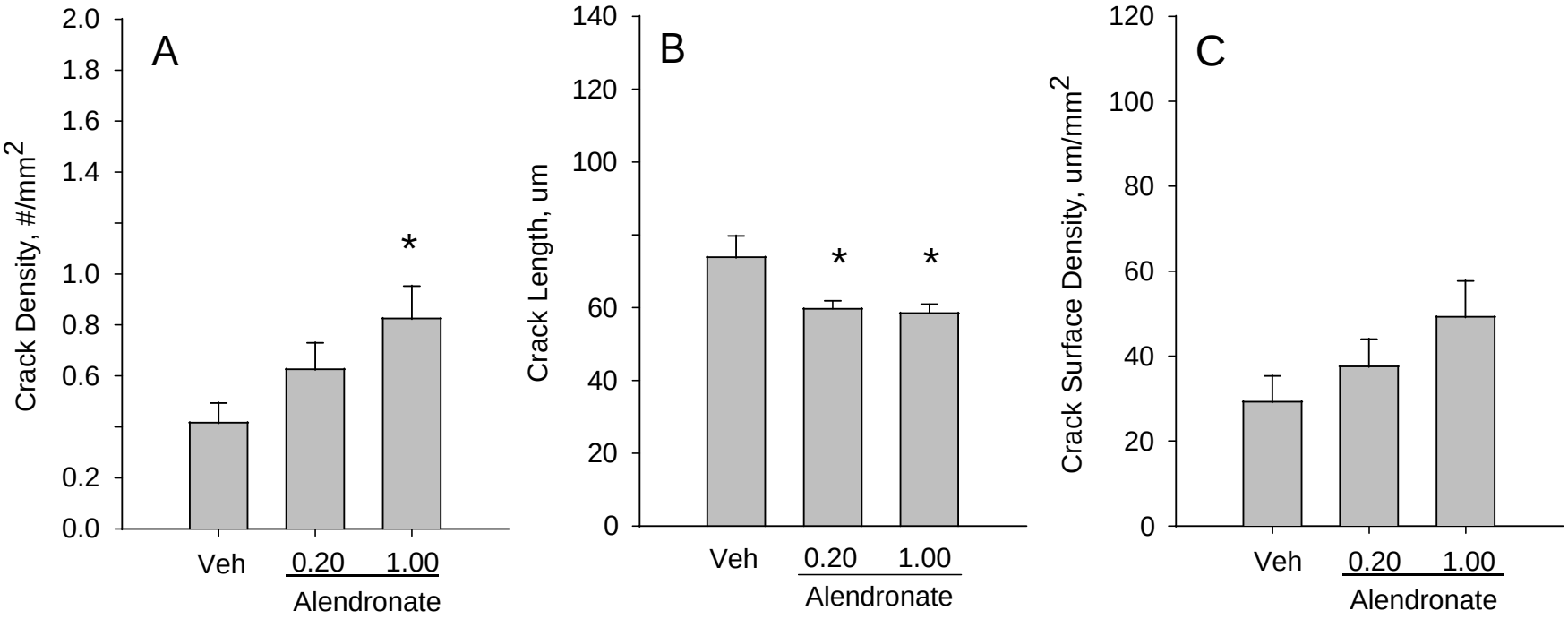

A
Crack Density, #/mm2
2.0
1.8
1.6
1.4
1.0
0.8
0.6
0.2
0.0
*
Veh
0.20
1.00
Alendronate
B
Crack Length, um
140
120
100
60
40
20
0
*
*
Veh
0.20
1.00
Alendronate
C
Crack Surface Density, um/mm2
120
100
80
60
40
20
0
Veh
0.20
1.00
Alendronate

Figure 2.

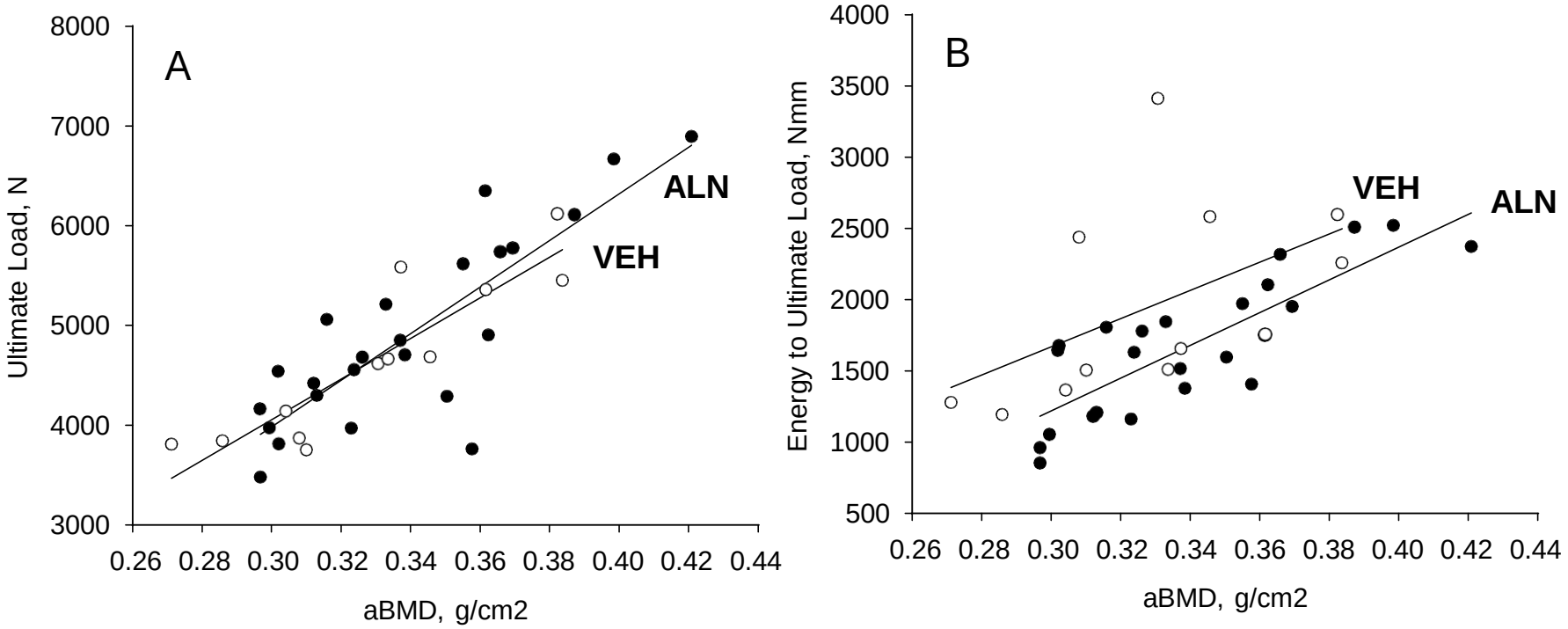
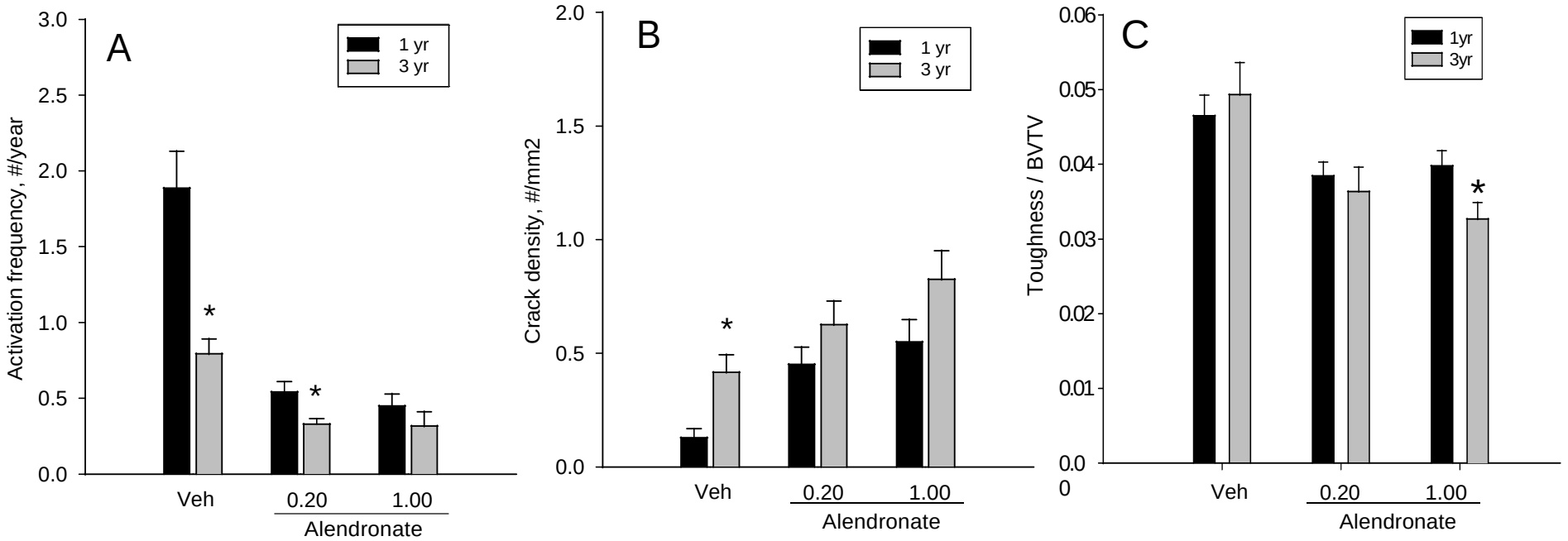

A
Ultimate Load, N
8000
7000
6000
5000
4000
3000
0.26 0.28 0.30 0.32 0.34 0.36 0.38 0.40 0.42 0.44
aBMD, g/cm2
ALN
VEH
B
Energy to Ultimate Load, Nmm
4000
3500
3000
2500
2000
1500
1000
500
0.26 0.28 0.30 0.32 0.34 0.36 0.38 0.40 0.42 0.44
aBMD, g/cm2
VEH
ALN

Figure 3.


A
1 yr
3 yr
Activation frequency, #/year
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Veh
0.20
1.00
Alendronate
B
1 yr
3 yr
Crack density, #/mm2
2.0
1.5
1.0
0.5
0.0
*
Veh
0.20
1.00
Alendronate
C
1yr
3yr
Toughness / BVTV
0.06
0.05
0.04
0.03
0.02
0.01
0.0
0
*
Veh
0.20
1.00
Alendronate